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## A single point mutation in the splice donor site of the low-density-lipoprotein-receptor gene produces intron read-through, exon-skipped and cryptic-site-utilized transcripts

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Familial hypercholesterolemia is a genetic disorder caused by mutations of the low-density-lipoprotein (LDL) receptor gene. We characterized the structures of LDL receptor mRNA transcripts in the fibroblasts of a homozygous patient carrying a single base substitution (T→C) at the 5' splice donor site of intron 12 of the LDL receptor gene. We identified three aberrant transcripts as a consequence of intron-12 read-through, exon-12 skipping and utilization of a cryptic splice donor site. Only a point mutation at the 5' splice donor site caused the production of three alternatively spliced products. None of these transcripts produced a functional LDL receptor protein in this patient.

**Keywords:** low-density-lipoprotein receptor; familial hypercholesterolemia; splicing mutation; read through.

Familial hypercholesterolemia (FH) is a genetic disorder characterized by an elevated level of low-density-lipoprotein (LDL) cholesterol, xanthomas and premature atherosclerosis [1]. FH is an inherited disease with an autosomal trait; the genetic basis of FH is a lack of functional receptors for LDL on the cell surface [1]. Various mutations of the LDL receptor gene have been reported including nonsense mutations, missense mutations, deletions and insertions [2, 3].

Hobbs et al. [4] described a Japanese homozygous FH patient who expressed two different-sized transcripts of the LDL receptor gene; the patient was proved to be a true homozygote carrying a point mutation at the splice donor site of intron 12 of the LDL receptor gene [3]. The sizes of the transcripts of the LDL receptor gene in this mutation were about 5.3 kb and 8.4 kb [3, 4]. Although the patient expressed an apparently normal-sized 5.3 kb transcript, she did not produce the LDL receptor protein that cross-reacted with the monoclonal antibody against the LDL receptor protein [4].

We have frequently found the identical mutation in Japanese FH patients: The patients, KIK, YS and MY [5], were true homozygotes for this mutation, whose phenotype belonged to the so-called 'null-allele' phenotype: neither LDL binding nor LDL receptor protein synthesis was detected in their fibroblasts.

According to Robberson et al. [6], the splice acceptor and donor sites of an exon are recognized and defined as a unit by the splicing machinery. Thus, a point mutation at a splice site leads to exon skipping and/or utilization of a cryptic splice site close to the site of mutation [6, 7].

As the patient with the mutation at the splice donor site of intron 12 did not produce the functional LDL receptor, the nor-

mal-sized transcript of 5.3 kb found in this patient [3, 4] is expected to result from skipping of exon 12 and/or utilization of a cryptic donor site in intron 12 close to the 5' splice site. The production of the 8.4-kb transcript, however, is hard to explain on the basis of the described rule [6, 7]. Therefore, in the present study, we analyzed the structure of the transcripts in the fibroblasts of this patient. We identified three aberrantly spliced transcription products; one contained the entire intron 12, one skipped exon 12, and one utilized a cryptic splice site in intron 12.

### MATERIALS AND METHODS

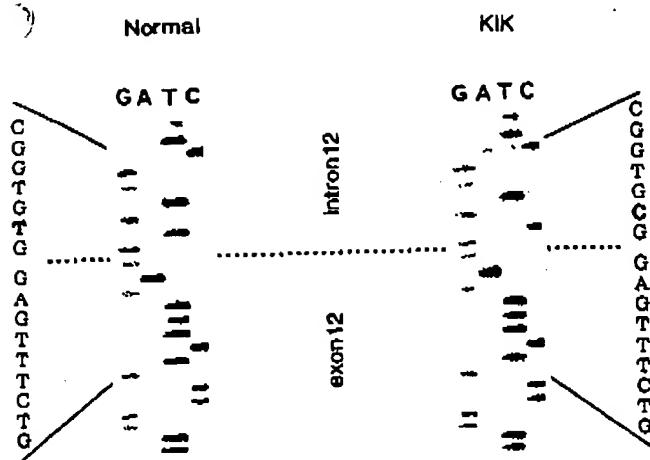
**Patient.** The proband, KIK, was a patient who visited our lipid clinic in the National Cardiovascular Center for precise examination of hypercholesterolemia. She was 4 years old and had severe hypercholesterolemia. As she was born from consanguineous marriage and her cultured skin fibroblasts showed a negligible amount of LDL receptor activity [5], she was diagnosed as a FH homozygote.

**Materials.** [ $\alpha$ -<sup>35</sup>S]dCTP (1000 Ci/mmol) was purchased from New England Nuclear. Nucleic acids and hexadecoxynucleotides were obtained from Pharmacia. NA45 membrane was from Schleicher and Schuell. The restriction enzymes were from Bethesda Research Laboratory, New England Biolabs, Takara Shuzo and Toyobo. The genomic clone, 233-2, which codes introns 10–15 of the normal LDL receptor gene, was obtained from the American Type Culture Collection.

**Amplification of genomic DNA.** Exons of the LDL receptor gene of the patient were amplified from 1 µg genomic DNA extracted from leucocytes by PCR with 2.5 units *Pfu* DNA polymerase (Stratagene) using an automated thermal cycler (Perkin-Elmer-Cetus). To amplify exons 1–17 and the coding region of exon 18 of the LDL receptor gene, the reported pairs of primers

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Abbreviations: LDL, low-density lipoprotein; FH, familial hypercholesterolemia; RT-PCR, reverse-transcription PCR.

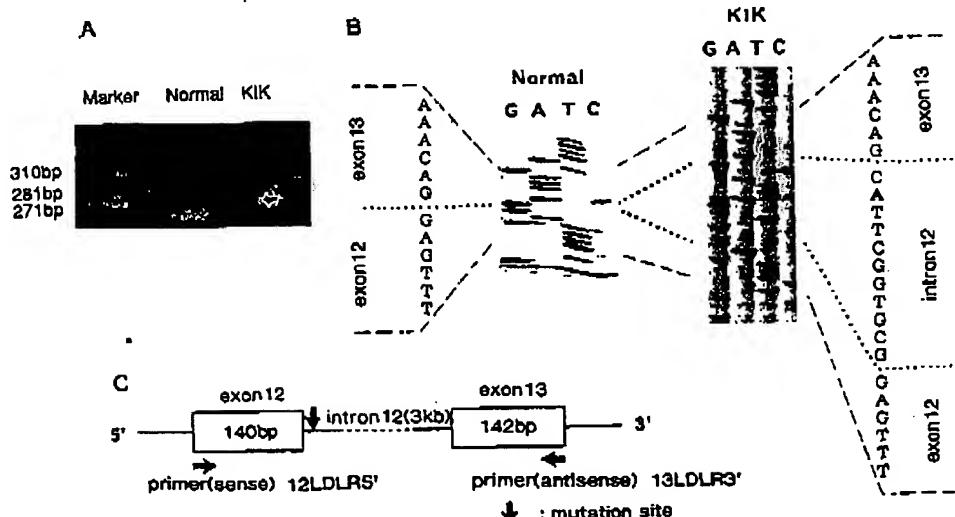


**Fig. 1.** DNA sequences across the 5' splice donor site of intron 12 of the LDL receptor gene from a normal subject and patient KIK. Samples of 1 µg genomic DNA from a normal subject and patient KIK were amplified by PCR. For the amplification reaction, oligonucleotides coding the intron sequence flanking the 5' side of exon 12 (12SP76) and the antisense sequence of the intron flanking the 3' side of exon 12 (12SP77) were used as primers. The amplified DNA was purified and subcloned into the *Sma*I site of pUC118, and the plasmid DNA was sequenced. The sequence revealed a single base change of T to C (letters in oblique) in the 5' splice donor site of intron 12. This base change was detected in all 12 subclones sequenced.

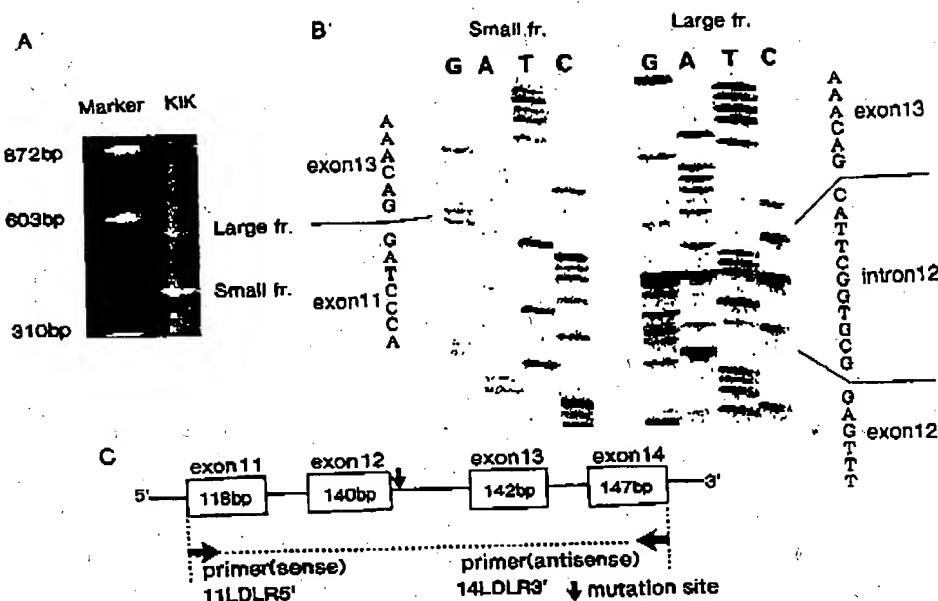
[8] were used, except that the two nucleotides at the 3' end of each oligonucleotide were omitted. Prior to the amplification, the oligonucleotide primers were treated with T4 polynucleotide kinase (Toyobo). The amplification reaction comprised 25 cycles of incubation of the reaction mixture for (a) denaturation at 94°C for 1.5 min, (b) annealing at 45–60°C for 2 min and (c)

extension at 72°C for 3 min. The amplified product was size fractionated by 2% agarose gel electrophoresis, then the band corresponding to each exon was adsorbed onto the NA4S membrane and eluted as described previously [9]. The purified DNA was blunt-ended with T4 DNA polymerase (Toyobo), subcloned into the *Sma*I site of pUC118 vector with T4 DNA ligase (BRL), amplified, then sequenced by the dideoxy method [10] using Sequenase (United States Biochemical).

**Amplification by reverse-transcriptase-coupled PCR (RT-PCR).** Total RNA was isolated by the guanidium thiocyanate method [11] from fibroblasts of a normal subject or the patient, followed by ultracentrifugation in a cesium chloride solution as described [12]. The RNA thus prepared was treated with DNase I (RNase-free, Takara Shuzo) in the presence of the RNase inhibitor (Takara Shuzo) [12]. Then, the RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer-Cetus) using random hexamers as primers, and the amplification was performed with 2.5 units *AmpliTaq* DNA polymerase (Perkin-Elmer-Cetus). A pair of sense and antisense primers was chosen from the following eight oligonucleotides and used for each RT-PCR. As the sense primer, an oligonucleotide was chosen from 11LDLR5' coding the 5' end of exon 11 (5'-TTCATGTACTGGACTGACTG-3'), 12LDLR5' coding the 5' end of exon 12 (5'-ATCTCCTCAGTG-GCCGCCTC-3'), and 13SP78 coding the intron sequence flanking the 5' end of exon 13 (5'-GTCATCTTCCTGCTGCCT-GTTT-3', coding nucleotides -25 to -3 from the beginning of exon 13) [8]. The antisense primer was one of 12LDLR32 coding the 5' side of intron 12 (5'-GTCTGTGTCTATCCGCCACC-3', coding nucleotides 51–32 from the beginning of intron 12), 13LDLR3' coding the 3' end of exon 13 (5'-CTCTGGCTGG-GTGAGGTTG-3'), 14LDLR3' coding the 3' end of exon 14 (5'-CTGTGAGGCAGCTCCTCATG-3'), 15LDLR3' coding the 3' end of exon 15 (5'-CTTGGTGAGACAGTTGCACT-3') and 16LDLR3' coding the 3' end of exon 16 (5'-CGATGGGGAGG-



**Fig. 2.** Analyses of the amplified DNA obtained by RT-PCR from the LDL receptor transcripts of a normal subject and patient KIK. (A) Polyacrylamide gel electrophoresis of the products of RT-PCR. Total RNA (1 µg) of a normal subject or patient KIK was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase using random hexamers as primers. The cDNAs thus prepared were amplified with *AmpliTaq* DNA polymerase. The sense primer, 12LDLR5', coding the 5' end of exon 12, and the antisense primer, 13LDLR3', coding the 3' end of exon 13, were used for the reaction. The products were electrophoresed in a polyacrylamide gel (20%), then stained with ethidium bromide. The fragment obtained from patient KIK was slightly larger than that obtained from a normal subject. (B) Nucleotide sequence of each fragment obtained by RT-PCR across the exon 12/13 junction. The fragments were purified, subcloned, then sequenced. In a normal subject, exon 12 was directly connected to exon 13. In patient KIK, 11 nucleotides at the beginning of intron 12 were inserted between exons 12 and 13. (C) Schematic illustration of the location of the mutation and the primers for RT-PCR.



**Fig. 3.** Analyses of the amplified DNA obtained by RT-PCR from patient KIK. (A) Polyacrylamide gel electrophoresis of the RT-PCR products. Total RNA (1 µg) from patient KIK was reverse transcribed, then PCR amplified. The sense primer, 11LDLR5', coding the 5' end of exon 11, and the antisense primer, 14LDLR3', coding the 3' end of exon 14, were used for the reaction. The products were electrophoresed in a polyacrylamide gel (20%), then stained with ethidium bromide. (B) Nucleotide sequences of the fragments. The two products were purified, subcloned, then sequenced. The small fragment (fr.) was produced by skipping of exon 12 (left panel). In the large fragment (right panel), the 11-nucleotide sequence of intron 12 was inserted, which was identical to that shown in Fig. 2B. (C) Schematic illustration of the location of the mutation and the primers for RT-PCR.

ACAATGGAC-3'). Prior to PCR, the primers were treated with T4 polynucleotide kinase. The amplification reaction comprised 35 cycles of incubation of the reaction mixture for (a) denaturation at 94°C for 1 min and (b) annealing and extension at 55°C for 1 min. The amplified product was size fractionated, subcloned, then sequenced as described above.

## RESULTS

The homozygous FH patient, KIK, was a 4-year-old female, whose clinical features have previously been described [5]. Her LDL receptor protein in cultured skin fibroblasts has been examined, and she has been classified as a so-called 'null-allele'-type homozygote [5].

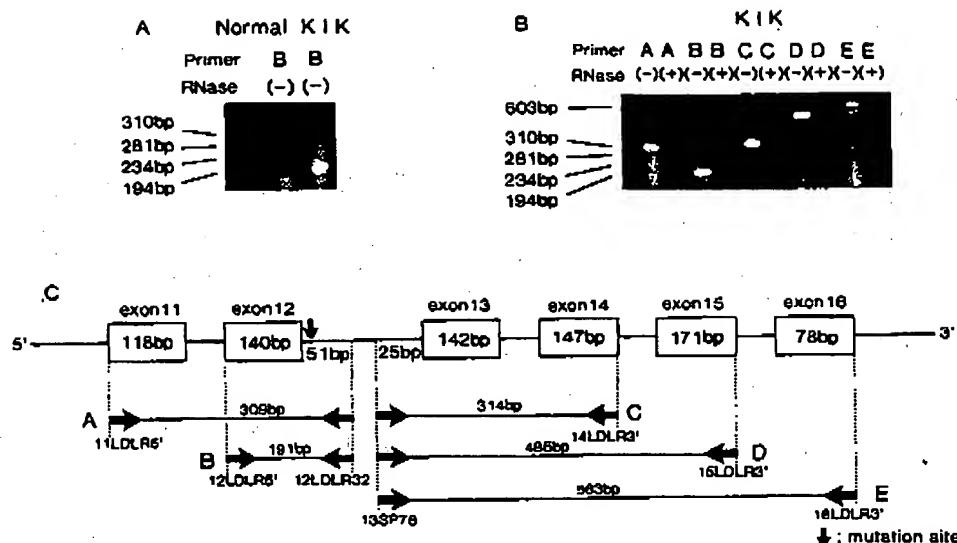
The sizes of the restriction fragments of her LDL receptor gene produced by digestion with EcoRI, *Bam*HII, EcoRV and *Xba*I were indistinguishable from those of a normal subject (data not shown). We amplified all 17 exons, the coding region of exon 18, and part of the 5' non-coding region of her LDL receptor gene by PCR, and the products were subcloned and sequenced. We could not find any abnormal sequence in the coding region and the 5' non-coding region of the patient's gene, except that two polymorphisms were found in the coding region: a C→T substitution at the third nucleotide of the codon for the Cys6 in exon 2 [13] and a T→C substitution at the third nucleotide of the codon for Asn570 in exon 12 [14]. Neither of them affected the coding amino acid sequence. Although we could not detect any abnormal sequence in the coding region of the patient, we found a T→C substitution at the second nucleotide of the 5' splice donor 12 site of intron 12 (Fig. 1). This one-base substitution was detected in all 12 subclones sequenced, suggesting that patient KIK was homozygous for this mutation.

As the detected mutation was at the base next to the antisense primer designed in intron 12, it was impossible to detect

the substitution- and/or deletion-including intron using this primer. In order to determine the further intron sequence of the patient, we planned to design a new antisense primer coding the downstream sequence of intron 12. To obtain the information on the normal sequence of intron 12, we subcloned the 9.4-kb fragment of genomic clone 233-2 and sequenced part of the fragment. We identified the sequence of 70 nucleotides of the 5' side of intron 12 (data not shown). Then, we synthesized a 20-residue oligonucleotide complementary to nucleotides 51–32 from the beginning of intron 12 (12LDLR32, 5'-GTCTGTGTCTATCCG-CCACC-3') and used it as an antisense primer for PCR. No additional base change was detected in the PCR-amplified fragment. As the mutant allele closely correlated to the plasma cholesterol level in her family (data not shown), this base substitution was expected to be the cause of FH in this family. This base substitution has been already reported in another Japanese FH patient by Hobbs et al. [3].

It has been reported that this mutation expresses two different-sized transcripts, an apparently normal-sized 5.3-kb transcript and one of 8.4 kb [3, 4]. In the present study, we attempted to analyze the structure of these transcripts. A point mutation in the 5' splice donor site often leads to exon skipping or utilization of a cryptic site in the following intron [6, 7]. First, in order to determine if a cryptic site was utilized for splicing, we carried out RT-PCR using a pair of primers designed in exons 12 and 13 (Fig. 2C). The amplified product of patient KIK was slightly larger than that of a normal subject (Fig. 2A). The sequencing of the RT-PCR product of the patient revealed that an extra fragment composed of 11 nucleotides was inserted between exons 12 and 13, which encoded the sequence at the beginning of the 5' region of intron 12 (Fig. 2B). Thus, in this mutation, a cryptic splice-donor site in intron 12 was utilized.

Next, we tested the possibility of exon 12 skipping. For this purpose, we used a pair of primers designed in exons 11 and 14



**Fig. 4.** Analyses of the amplified products of RT-PCR from a normal subject and patient KIK. (A) Agarose gel electrophoresis of the RT-PCR products. Total RNA (1 µg) from a normal subject or patient KIK was reverse transcribed, then PCR amplified. The sense primer, 12LDLR5', coding the 5' end of exon 12, and the antisense primer, 12LDLR32, coding the intron sequence from nucleotides 51–32 in intron 12, were used for the reaction. The products were electrophoresed in agarose gel (2%), then stained with ethidium bromide. An amplified fragment was observed in patient KIK, but no product was observed in the normal subject. (B) Agarose gel electrophoresis of RT-PCR products from patient KIK. The pairs of primers used for the amplification were as follows: A, the sense primer, 11LDLR5', coding the 5' end of exon 11, and the antisense primer, 12LDLR32, coding the sequence of intron 12; B, the sense primer, 12LDLR5', coding the 5' end of exon 12, and the antisense primer, 12LDLR32; C, the sense primer, 13SP78, coding the intron sequence flanking the 5' end of exon 13, and the antisense primer, 14LDLR3', coding the 3' end of exon 14; D, the sense primer, 13SP78, and the antisense primer, 15LDLR3', coding the 3' end of exon 15; E, the sense primer, 13SP78, and the antisense primer, 16LDLR3', coding the 3' end of exon 16. Prior to RT-PCR, template RNA was treated with (+) or without (−) RNaseA. The products of RT-PCR were electrophoresed in an agarose gel (2%), then stained with ethidium bromide. The amplified bands were not observed for the samples treated with RNaseA. (C) Schematic illustration of the location of the mutation and the primers for RT-PCR.

**Table 1.** The scores of the splice donor and acceptor sites for introns 10–13. The scores were calculated according to the methods of Shapiro and Senapathy [18].

Splice sites	Nucleotide position	Nucleotide sequences	Score
5'			
consensus		AG/GT <sup>RA</sup> GT	100.0
normal	intron 11 +1	AG/GTATGT	88.7
	intron 12 +1	AG/GTGTGG	80.8
	intron 13 +1	AG/GTAAGG	95.4
mutant	intron 12 +1	AG/GCGTGG	62.6
cryptic 1	intron 12 +3	GC/GTGGCT	50.4
cryptic 2	intron 12 +12	AC/GTACGA	69.3
cryptic 3	intron 12 +33	AG/GTGGCG	67.2
3'			
consensus		YYTTYYYYYYNCAG/G	100.0
intron 10		TGTCCCTCCCACCAAG/G	86.9
intron 11		ACTTGTCGTCTAG/G	73.6
intron 12		TGCTGCGCTGTTAG/G	80.6

(Fig. 3C). Two different-sized fragments were amplified from the transcripts of patient KIK with this pair of primers (Fig. 3A). The large-sized fragment contained 11 extra nucleotides of intron 12, the sequence of which was identical to that shown (Fig. 2B). The small-sized fragment was the product of exon 12 skipping, exon 11 being directly connected to exon 13 (Fig. 3B).

The small-sized fragment was the major product of the amplification, showing that exon skipping was more apt to occur than utilization of a cryptic site in this mutation.

Neither exon skipping nor utilization of a new cryptic site can explain the size of the reported 8.4-kb transcript. Although it does not coincide well with the proposed mechanism of splicing [6], the most feasible explanation for the production of the 8.4-kb transcript is that only intron 12, which is about 3 kb in size, failed to be spliced. To determine if a 'read-through' product exists, we designed five pairs of primers for RT-PCR (Fig. 4). When the pairs of primers A and B were used for RT-PCR, 309-bp and 191-bp fragments, respectively, were amplified for transcripts from the patients (Fig. 4A and B). Sequence analysis confirmed that the 309-bp fragment encoded the sequences of exons 11 and 12 and a part of intron 12, and that the 191-bp fragment encoded those of exon 12 and a part of intron 12, as expected (data not shown). The expressed transcript from the patient did not contain intron 11, which was normally spliced out, but it contained the sequence of intron 12, which failed to be spliced out. Using the primers C, D and E, fragments of 314, 485 and 563 bp, respectively, were amplified for transcript from the patient (Fig. 4B). The sequencing of these fragments (data not shown) clearly indicated that the expressed transcript did not contain the sequences of introns 13, 14 and 15, which were normally spliced out, but contained the 3' region of intron 12 which failed to be spliced out. From the above data, we can conclude that the patient produced a transcript in which only intron 12 failed to be spliced out. The expected size of the transcript of the entire intron 12 is about 3 kb, which may explain the size (8.4 kb) of the larger transcript observed in this mutant [3, 4]. As the normal subject did not exhibit any amplified frag-

A Read through	exon 12	intron 12	
mutant allele	TTT GAG GCG TGG CTT ACG TAC GAG ATG CAA GCA		
	Phe Glu Ala Trp Leu Thr Tyr Glu Met Gln Ala		
B Exon skipping	exon 11	exon 12	exon 13
normal allele	ACC CTA G	AT CTC	TGT GAG GAC AAA
	Thr Leu Asp Leu		Phe Glu Asp Lys
mutant allele	ACC CTA G	GA CAA	GTT GGC TGA
	Thr Leu Gly Gln		Val Gly Stop
C Alternative splicing	exon 12	exon 13	exon 14
normal allele	TTT GAG GAC AAA	AGA G	GA GTG
	Phe Glu Asp Lys	Arg	Gly Val
mutant allele	TTT GAG GCG TGG CTT AC	G ACA AAG	CAA GAG GAG TGA
	Phe Glu Ala Trp Leu Thr	Thr Lys	Gln Glu Glu Stop

Fig. 5. Schematic illustration of the possible alternative translation products resulting from the mutation. (A) The 'read-through' product lacks splicing of intron 12. Translation of this 'read-through' transcript is expected to continue into intron 12. We could not detect the presence of putative stop codons within 70 nucleotides of the 5' side of intron 12, thus translation might continue further into intron 12. (B) The product of 'exon-skipping' lacks the entire exon 12, exon 11 being directly connected to exon 13. The expected translation may produce a frame-shifted protein, and translation may terminate at a putative in-frame stop codon which appears in exon 13. (C) The product of 'alternative splicing' utilizes the cryptic GT site within intron 12. Translation may terminate at a putative in-frame stop codon which appears in exon 14.

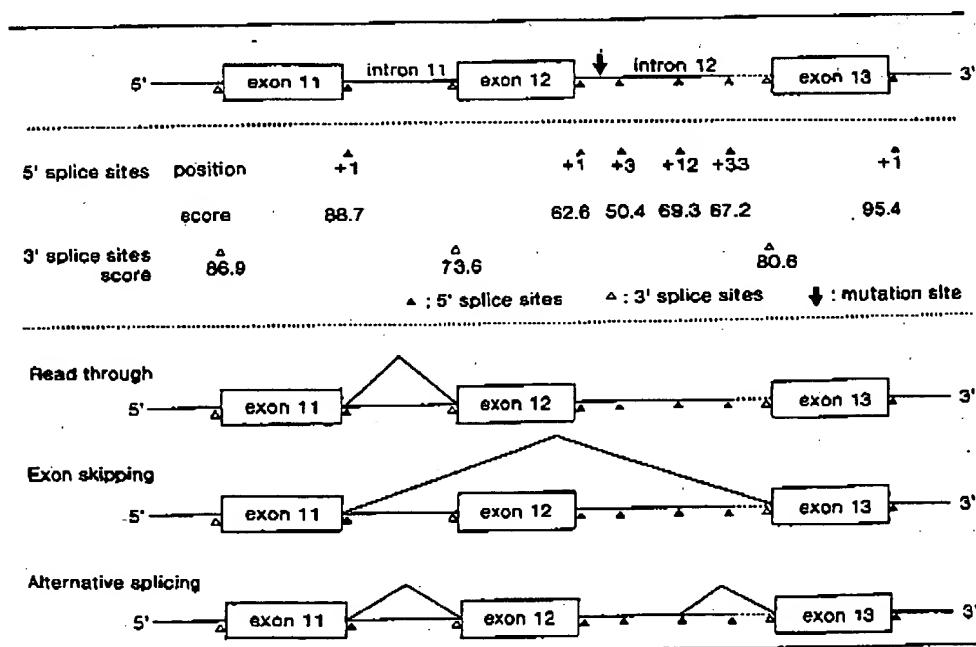


Fig. 6. Schematic illustration of the splice-site scores and the locations of the possible splice junctions in the mutant. Open triangles indicate 3' splice acceptor sites. Closed triangles indicate possible 5' splice donor sites. The arrow indicates the mutated site in patient K/K. The splice-site scores for the mutant are indicated under the symbols.

ment on RT-PCR when the primers were designed in intron 12 (a typical example is shown in Fig. 4 A), and RNase A treatment of patient RNA prior to the reverse transcription wiped out the PCR product (Fig. 4 B), the amplified fragments were not derived from the contaminated genomic DNA.

Only one base substitution in the 5' splice donor site of intron 12 resulted in the production of three aberrant transcripts

due to (a) intron read-through, (b) exon-skipping and (c) utilization of a cryptic donor site (Fig. 5).

## DISCUSSION

In the present study, we identified a single base substitution (T-C) in the 5' splice donor site of intron 12 of the LDL recep-

tor gene in a Japanese homozygous FH patient, KIK. This mutation was identical to that already reported by Hobbs et al. [3]. In the present study, we identified three different transcripts caused by this point mutation; one containing intron 12 (intron read-through), one lacking exon 12 (exon-skipping) and one with an extra fragment composed of 11 nucleotides of the 5' region of intron 12 due to utilization of a cryptic splice donor site (alternative splicing; Fig. 5). As we could not detect synthesis of the LDL receptor protein in the patient [5], none of the transcripts are expected to produce the functional LDL receptor protein. There are three possibilities; translation in these aberrant transcripts is unsuccessful; the translation products from these transcripts are extremely unstable, or the translation products are not recognized by the antibody.

In general, a mutation at the second nucleotide of the GT dinucleotide of the 5' splice donor site leads to aberrant splicing [7, 15–17]. Such a mutation often produces a transcript which skips the upstream exon or which utilizes a cryptic 5' donor site usually located in the following intron [6, 7, 17]. In patient KIK, both types of transcripts were identified. Skipping of exon 12 was more apt to occur than utilization of the cryptic splice donor site in intron 12 (see Fig. 3A).

We scored the potentiality of the splice donor and acceptor sites of introns 10–13 by the procedure of Shapiro and Senapathy [18] (Table 1). The scores for the original and the mutated 5' splice donor sites were calculated to be 80.8 and 62.6, respectively. The cryptic splice donor site which was actually utilized in the mutant was scored as 69.3 (cryptic 2 in Table 1), which is slightly higher than that of the mutation site. We searched for possible cryptic sites and found the two potential splice donor sites within 70 nucleotides of the 5' end of intron 12. One was at position 3 from the beginning of the intron and scored as 50.4 (cryptic 1 in Table 1), the other was at position 33 and scored as 67.2 (cryptic 3 in Table 1). The scores for these two sites were lower than that of the actually selected splice site (Fig. 6). A mutation in a splice donor site often skips the upstream exon of the mutation site [7] when it is hard to find a suitable cryptic site in the region close to the mutation site. This is clearly explained by the fact that the splicing machinery recognizes the 5' splice donor site and the 3' acceptor site of an exon as a unit [6]. Since the score of the cryptic splice donor site in intron 12 was lower (69.3) than that of the original site (80.8), skipping of exon 12 was more apt to occur than utilization of a cryptic site (Fig. 4A).

Both exon 12 skipping and utilization of a cryptic splice donor site can only explain the transcript of about 5.3 kb [3, 4]. We expected that the 8.4-kb transcript, which was another aberrant transcript observed in the mutant, was produced by failure of splicing out of intron 12. We identified the transcript in which introns 11, 13, 14 and 15 were normally spliced out, but intron 12 was not (Fig. 4). Exon 12, together with intron 12, might be recognized as a large exon. This could be the reason why the 8.4-kb transcript was observed in the mutant [3, 4]. Generally, however, a large exon comes, without exception, at the end of a gene. When an exon is larger than 300 bp, splicing components cannot recognize both ends of an exon [6]. Therefore, at present, we cannot explain the mechanism by which the transcript, including the entire intron 12 which is about 3 kb in size, was produced, and why such a large 'pseudo-exon' can exist in the middle of the transcript. This aberrant transcript might be a transient form of the splicing product, on which the splicing components are stacked, and the transcript might remain inside the nucleus.

The point mutation described in the present study is interesting for understanding splicing mechanisms.

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